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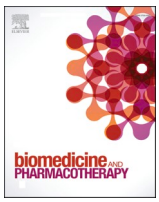

# PPARγ antagonism as a new tool for preventing or overcoming endocrine resistance in luminal A breast cancers

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ABSTRACT

*Purpose:* This research investigates the role of PPARγ in the complex molecular events underlying the acquisition of resistance to tamoxifen (Tam) in luminal A breast cancer (BC) cells. Furthermore, it focuses on evaluating the possibility of repurposing Imatinib mesylate, an FDA-approved anticancer agent recently recognized also as a PPARγ antagonist, for the personalized therapy of endocrine-resistant BC with increased PPARγ expression.
*Methods:* Differential gene expression between parental and Tam-resistant MCF7 cells was assessed by RNA-seq followed by bioinformatics analysis and validation by RT-qPCR. PPARγ was downregulated by esiRNAs or inhibited by the antagonist GW9662. Cell viability and proliferation were measured by MTT and colony formation assays. Spheroids were prepared from parental and Tam-resistant MCF7 cells. Other luminal A BC cell lines resistant to Tam were generated.
*Results:* In MCF7-TamR cells, PPARγ and several of its target genes were significantly upregulated. Increased PPARγ expression was due to the modulation of its positive/negative transcriptional regulators. Downregulating PPARγ with esiRNAs or GW9662 effectively killed parental and Tam-resistant cells and spheroids. Imatinib revealed to be as effective as GW9662 in restoring Tam susceptibility of these cells. PPARγ overexpression was also observed in the newly-selected Tam-resistant luminal A BC cells, in which GW9662 and Imatinib restored their susceptibility to Tam.
*Conclusion:* Our findings demonstrate that the overexpression of PPARγ is a frequent occurrence during acquisition of Tam resistance in luminal A BC cells, and that PPARγ antagonism represents an alternative therapeutic approach for the personalized treatment of BC showing dysregulation of this nuclear receptor.

## 1. Introduction

Breast cancer (BC) is the most prevalent cancer in women worldwide [1]. This cancer is quite heterogeneous, both from the molecular and the clinical point of view. Based on the expression of a panel of 50 key genes [2], it is classified under five major intrinsic subtypes, which are the luminal A, luminal B, Her2-enriched, claudin low, and triple-negative breast cancer (TNBC) [3]. The latter is a heterogeneous group of BC currently further divided in several molecular subtypes based on their specific genomic-transcriptomic, immunohistochemical or metabolic signature [4–6]. Luminal A and B subtypes account for about 70 % of total cases and are endowed with a better prognosis. The choice of the first-line therapeutic approach is mostly dictated by the specific subtype [3,7]. For example, those expressing estrogen receptor (ER) are eligible for the endocrine therapy aimed at restraining the transduction of hormonal signals through the ERα or β. Among the selective modulators of ER activity, tamoxifen (Tam) represents the standard treatment. Differently, the HER2-overexpressing forms are treated with anti-HER2 monoclonal antibodies or antibody/drug conjugates. By contrast TNBC account for about 12–17 % of cases and express neither estrogen/progesterone receptors nor Her2, which limits the therapeutic opportunities. However, in addition to the conventional chemotherapy, the TNBC may benefit of targeted therapies which have been devised based on their specific molecular, biological and metabolic peculiarities (reviewed in [5,6]).

Despite the current therapeutic protocols have significantly improved the treatment outcome, about 30 % of BC patients develops resistance to anticancer treatments, which accounts for relapse and disease progression [8]. Several molecular events have been recognized to contribute to the acquisition of resistance, such as an altered hormone receptor expression or signaling, dysregulated autophagy, and reprogramming of lipid metabolism [9,10]. The latter has recently emerged as

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one of the new hallmarks of tumor progression, including the acquisition of drug resistance [11].

Peroxisome Proliferator-Activated Receptors (PPARs) are transcription factors activated by both natural or synthetic ligands mainly involved in the regulation of lipid and carbohydrate metabolisms. In humans, PPAR family includes three isoforms, PPARα, PPARβ/δ and PPARγ, which show tissue-specific distribution and modulate several other processes in addition to lipid metabolism, such as cell proliferation, survival, differentiation, inflammatory and immune responses [12]. PPAR expression is controlled at both transcriptional and post-transcriptional levels. The transcriptional regulators encompass some of the Master Regulator Transcription Factors (MRTFs), including ELF, KLF, GATA [13] and many miRNAs [14,15]. As an instance, during adipogenesis PPARγ is overexpressed due to the increase of the positive regulators KLF5 and EBF1. Moreover, the activity of PPARs is regulated at the post-translational level by phosphorylation, SUMOylation, ubiquitination and acetylation [12]. In the presence of ligands, PPARs heterodimerize with nuclear retinoid X receptor (RXR) and bind the Peroxisome Proliferator Response Elements (PPRE) within the promoter of target genes [16], for which the PPARs may thus act as direct activators. However, it has been also clarified that PPARs can specifically restrain the expression of certain genes by two main mechanisms indicated as ligand-dependent transrepression and ligand-independent repression [17].

Based on the multiplicity of their functions, the role of PPARs in cancer development and progression has been extensively studied. In BCs, both increased [18] or decreased [19] PPARγ expression has been reported, which makes the role of this family of nuclear transcription factors in cancer development or progression quite ambiguous. The bioinformatics analysis of TCGA and GTEx public datasets has evidenced that the expression of PPARγ is globally decreased in specimens of different BC subtypes compared to the normal tissue [19] and that, among the different subtypes, its expression is higher in ER-positive than in ER-negative BCs. The actual involvement of PPARγ [20] in the onset of drug resistance in BC is presently far from fully elucidated at both the experimental and preclinical levels, mainly in consequence of the paucity of the investigations performed so far. An in vitro study evidenced that combining Tam and troglitazone, a specific PPARγ agonist, improved the cytotoxic effect of the drug through the downregulation of cyclin D1 [21]. The beneficial effect of troglitazone was not confirmed by a clinical trial performed on BC patients refractory to both hormonal therapy and conventional chemotherapy, which failed to demonstrate that this PPARγ agonist was able to stop the progression of the disease [22]. In other studies on malignant mammary lesions induced by DMBA, the inhibition of transcriptional activity of PPARγ achieved by the selective antagonist GW9662 was shown to restore susceptibility to Tam treatment [23]. More recently, PPARγ has been reported to mediate FABP5-induced resistance to doxorubicin in both resistant cell lines and patients, suggesting its possible role in acquiring drug resistance [24].

In this research, we have explored the regulation of PPARγ and its involvement in the onset of endocrine resistance in a Tam-resistant (TamR) subline of MCF7 cells as well as in a panel of other BC cells of the luminal A subtype. Moreover, we have investigated the effects of interfering with the expression or the activity of this transcription factor on survival and Tam resistance of the above BC cells. Moreover, we have also focused on testing the possibility of repurposing Imatinib mesylate, demonstrated to act also as a PPARγ antagonist [25], for treatment of advanced luminal A BCs with dysregulated PPARγ.

## 2. Materials and methods

### 2.1. RNA sequencing and bioinformatics analysis

Total RNA was extracted from MCF7 and MCF7-TamR cells with the ReliaPrep™ RNA Miniprep System (Z6010, Promega, Milan, Italy) and used for strand-specific library preparation and RNA sequencing (RNA-seq) at Novogene (Cambridge, UK). RNA sequencing was performed with the Illumina platform; the resulting reads were aligned to the GRCh38 reference human genome using STAR software (version 2.5) to obtain the read counts for the downstream analyses. The R analytical software (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) under the Rstudio graphical user interface was used to analyze the raw data. Differential gene expression analysis was performed with DESeq2 package (ver. 1.40.2) in Bioconductor (ver 3.17). All the differentially expressed genes that were upregulated in MCF7-TamR cells were further analyzed for both Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways enrichment with the Enrichr Analysis Tool [26]. The top 10 most significantly enriched terms (GO) and pathways (KEGG) were visualized with the R package ggplot2.

TCGA clinical data were accessed through the cBioportal (https://www.cbioportal.org/). For the present investigation, the TCGA Pan-Cancer Atlas dataset made of 1048 samples of breast invasive carcinomas was used for the quantification of the PPARγ mRNA using the cBioportal query and data presentation functions. The reanalysis of RNA-seq data of human BC circulating tumor cells, metastases and the relevant control samples [27] was performed with the R analytical software on the samples included in the GSE113890 dataset downloaded from the public repository GEO (https://www.ncbi.nlm.nih.gov/geo/).

### 2.2. Cell cultures

MCF7, MDA-MB-415, T47D and ZR-75-1 cells were grown in DMEM (D6429, Merck, Darmstadt, Germany) supplemented with 10 % FBS (F7524, Merck), 100 U/mL penicillin and 100 μg/mL streptomycin (P0781, Merck) at 37 °C in a humidified atmosphere containing 5 % $CO_2$. 1 % nonessential amino acids were added to the culture medium of ZR-75-1 and ZR-75-1-TamR cells. Tam-resistant sublines of MDA-MB-415, T47D and ZR-75-1 cells were generated by growing the parental cells in the presence of increasing concentrations of the drug (sc-208414, Santa Cruz Biotechnology, Heidelberg, Germany) as described for MCF7-TamR cells [10], and designated by adding the suffix '-TamR' to the name of the parental counterpart. All the Tam-resistant clones were routinely maintained in the presence of 5 μM Tam in the growth medium.

### 2.3. Semiquantitative real-time RT-qPCR

Total RNA was extracted with the TriReagent (T9424, Merck); 1 μg of total RNA from each sample was reverse transcribed with the FireScript RT cDNA synthesis Kit (06–12–00200, Solis BioDyne, Tartu, Estonia). A volume of cDNA corresponding to 50 ng of total RNA/sample was amplified in a CFX Connect (Bio-Rad Laboratories, Hercules, CA, USA) with the HOT FIREPol Evagreen qPCR Supermix (08–36–00001, Solis BioDyne). The PCR primers used for the amplifications (Table 1) were designed with Primer 3 except those for GPX3, SOD1 and VEGFA that were obtained by PrimerBank [28]. The relative mRNA content was calculated using the $2^{-\Delta\Delta CT}$ method.

### 2.4. Western blotting

For western blotting analysis, the cells were detached by trypsinization and collected by centrifugation at 600 x g for 10 min. The samples were homogenized by sonication for 10 sec in ice-cold RIPA buffer; 30 μg of total lysates were separated on a 6–15 % gradient polyacrylamide gel and blotted to a nitrocellulose membrane. Probing of the membranes with an anti-PPARγ (sc-7273, Santa Cruz Biotechnology) or anti-β-actin (A5441, Merck) was performed as described [10]. The bands were revealed by incubation with Clarity ECL Western Blotting Substrate (1705061 Bio-Rad Laboratories) and detected with the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories). The optical density of the bands was measured with Image Lab (Bio-Rad Laboratories); the relative

**Table 1**
Primer pairs used for the Real-Time qRT-PCR.

| Target | PrimerBank ID | Forward | Reverse |
|---|---|---|---|
| CAT | - | TGTTGCTGGAGAATCGGGTTC | TCCCAGTTACCATCTTCTGTGTA |
| EBF2 | - | GGAATGTCGGAGTGGTGGA | TGTCATAGAGCGCCAGGAC |
| GAPDH | - | CGGGAAACTGTGGCGTGATG | ATGCCAGTGAGCTTCCCGTT |
| GATA3 | - | CACAACCACACTCTGGAGG | GGTTTCTGGTCTGGATGCC |
| GPX3 | 89903006c1 | AGAGCCGGGGACAAGAGAA | ATTTGCCAGCATACTGCTTGA |
| HMGCS2 | - | CGTCCCGTCTAAAGGTGTTCT | CGCTAGAGATGGCTCCTCAC |
| KLF2 | - | CCAAGAGTTCGCATCTGAAGGC | CCGTGTGCTTTCGGTAGTGG |
| KLF5 | - | CCGTCACCACCAAGCTCAGA | CTGGCAGGGTGGTGGGTAAA |
| PLIN2 | - | ATGGCATCCGTTGCAGTTGAT | GGACATGAGGTCATACGTGGAG |
| PPARγ | - | TACTGTCGGTTTCAGAAATGCC | GTCAGCGGACTCTGGATTCAG |
| SCARB1 | - | GGTCCAGAACATCAGCAGGATC | GCCACATTTGCCCAGAAGTTCC |
| SOD1 | 48762945c1 | GGTGGGCCAAAGGATGAAGAG | CCACAAGCCAAACGACTTCC |
| VEGFA | 284172466c1 | AGGGCAGAATCATCACGAAGT | AGGGTCTCGATTGGATGGCA |

amount of PPARγ protein was calculated by normalizing the optical density of the specific band against that of β-actin, used as a loading control.

### 2.5. Viability assay

Cell viability was determined with the MTT test. All the parental and Tam-resistant cell lines were seeded at 3 or 3.5 $\times 10^4$ cells/cm$^2$, respectively, in 100 μL of the appropriate growth medium; after treatments, 20 μL of an MTT solution (5 mg/mL; M2128, Merck) were added to each well for 2 h. The formazan precipitates were dissolved in 100 μL of DMSO by shaking the plates on an orbital shaker for 30 min at room temperature before measurement of the absorbance at 595 nm with an iMark Microplate Reader (Bio-Rad Laboratories).

### 2.6. Downregulation of PPARγ

Silencing of PPARγ was achieved by incubating the cells for 96 h with 120 nM of the predesigned MISSION esiRNAs (EHU097711; Eupheria Biotech, Merck, Milan, Italy) to minimize the off-target effects. The esiRNAs were delivered to the cells by Lipofectamine RNAiMAX (13778100, Thermo Fischer Scientific, Waltham, MA, USA); the effectiveness of silencing was verified by Real-Time RT-qPCR and western blotting after 36 h of incubation with the esiRNAs.

### 2.7. Colony formation assay

MCF7 and MCF7-TamR cells were seeded in 48-well plates (Jet Biofil, Alicante, Spain) at 100 cells/well in 0.25 ml of complete growth medium and allowed to adhere overnight under standard growth conditions. The day after seeding, the cells were treated with different concentrations of GW9662 (sc-202641, Santa Cruz Biotechnology) or Imatinib mesylate (sc-202180, Santa Cruz Biotechnology: from therein after, Imatinib) and allowed to form colonies for 14 days without any medium change. Subsequently, the colonies were simultaneously fixed and stained for 30 min with 0.1 % (w/v) crystal violet in 20 % methanol, washed with distilled water, and counted manually.

### 2.8. Determination of spheroid viability

For spheroid formation, MCF7 and MCF7-TamR cells were seeded in 24-well plates in a fibrin gel. To obtain the fibrin gel, 0.125 mL of an 8 mg/mL fibrinogen solution prepared in T7 buffer (50 mM Tris, 150 mM NaCl, pH 7.4) was mixed with an equal volume of cell suspension prepared at 4000 cells/mL. A volume of 0.25 mL of the mixture of fibrinogen solution and cell suspension was seeded into each well already containing 5 μL of thrombin (0.1 UI/μL); the gel was allowed to form overnight at 37 °C. The next day, 0.25 mL of medium were added on the top of the fibrin gel layer, after which the spheroids were allowed to form for 10 days. Subsequently, the spheroids were treated for up to 96 h with different concentrations of GW9662 or Imatinib. The morphology and the size of the spheroids were monitored daily for additional 4 days until the appearance of overt signs of degeneration of the spheroids exposed to the drugs. Viability of the spheroids was assessed after 96 h of drug treatment by adding to the wells a solution of propidium iodide (2 μg/mL final concentration) for 15 min. After staining, the spheroids were imaged with a Zeiss Axiovert 35 inverted microscope; phase contrast and fluorescence images were overlaid with ImageJ.

### 2.9. Statistical analysis

Data represent the mean ± SD of three independent experiments performed in triplicate. Differences between groups were assessed with either one-way ANOVA followed by the Student-Newman-Keuls or the Dunnet's post-*hoc* tests or Student's *t*-test using the Instat package (GraphPad Software, Boston, MA, USA). A $p$-value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Expression of PPARγ is increased in Tam-resistant breast cancer cell lines

RNA-seq analysis of MCF7 and MCF7-TamR cells revealed that the acquisition of Tam resistance markedly impacts gene expression. This altered gene regulation gives rise to the formation of two well-separated transcriptional clusters in the principal component analysis plots (Fig. S1A), suggestive of a well-differentiated gene expression repertoire in the frame of a substantial coherence among the biological replicates analyzed for each cell line. The transcriptome analysis showed that 12,303 genes were coexpressed by both cell lines, and that 766 and 656 were unique for MCF7 or MCF7-TamR cells, respectively (Fig. S1B). The analysis also revealed that a total of 8706 genes were differentially expressed, 4428 of which (50.9 %) were upregulated and 4278 (49.1 %) downregulated in MCF7-TamR cells compared with the parental counterpart (Fig. S1C). To detect the biochemical pathways and the subcellular compartments most affected by the acquisition of endocrine resistance, GO and KEGG analyses were further performed on the upregulated genes. According to the 2023 update of the Enrichr Reactome, the genes involved in lipid metabolism (in particular of fatty acids), protein phosphorylation/dephosphorylation or associated with subcellular compartments such as endosomes, mitochondria and peroxisomes were highly represented among the top-10 most significantly enriched terms in the biological processes and cellular compartment, respectively (Fig. S2). A significant role for genes involved in both lipid biosynthesis and degradation emerged also from the KEGG analysis of genes upregulated in MCF7-TamR cells. In fact, three among the top-10 enriched terms (fatty acid degradation, biosynthesis of unsaturated fatty acids and fatty acid elongation) encompass genes partaking in fatty acid

metabolism.

The analysis also evidenced that PPARγ was one of the most significantly upregulated genes in MCF7-TamR cells (Log2FC = 2.55; p-adj = $1.15 \times 10^{-198}$; Fig. 1A). Given its established role in the control of lipid metabolism [29], we further verified the RNA-seq data on PPARγ overexpression, before investigating its potential role in contributing to Tam-resistance of BC cells. RT-qPCR assays definitely confirmed that MCF7-TamR cells largely overexpress PPARγ mRNA (Fig. 1B) and protein (Fig. 1C-D) compared with the parental cell line. Since PPARγ is known to be translated into two isoforms, PPARγ-1 and 2, we compared the migration pattern of PPARγ bands from our samples with those from 3T3-L1 cells, which express both isoforms. The results evidenced that both MCF7 and MCF7-TamR cells only express the smaller isoform 1 of the protein, though at very different levels (Fig. 1E).

### 3.2. PPARγ upregulation in MCF7-TamR cells relies on an altered transcriptional control

The expression of PPARγ gene is strictly controlled at the transcriptional level. To verify whether PPARγ overexpression in MCF7-TamR cells was ascribable to a dysregulated gene transcription, we first checked the expression of the PPARγ regulators in our transcriptomic data. The search evidenced a coordinated modulation of the transcription factors accounting for either the positive (EBF2 and KLF5) or negative (GATA3 and KLF2) regulation of PPARγ (Fig. 2A). In fact, the transcriptome analysis (Fig. 2A) and the subsequent validation of some of the most relevant transcripts (Fig. 2B) by RT-qPCR showed that the expression of EBF2 and KLF5, which favor PPARγ expression, was significantly increased in MCF7-TamR cells, while that of KLF2, GATA3 and TRIB3, which account for its repression, was decreased or unchanged, respectively.

### 3.3. PPARγ-target genes are upregulated in MCF7-TamR cells

We subsequently focused on evaluating if also the transcriptional activity of PPARγ was higher in MCF7-TamR than in parental cells. To this aim, the transcriptomic data were screened again to verify the expression of a set of genes known to be regulated by PPARγ [16]. RNA-seq data analysis revealed that several among the validated PPARγ target genes were overexpressed in Tam-resistant cells (Fig. 3A). These

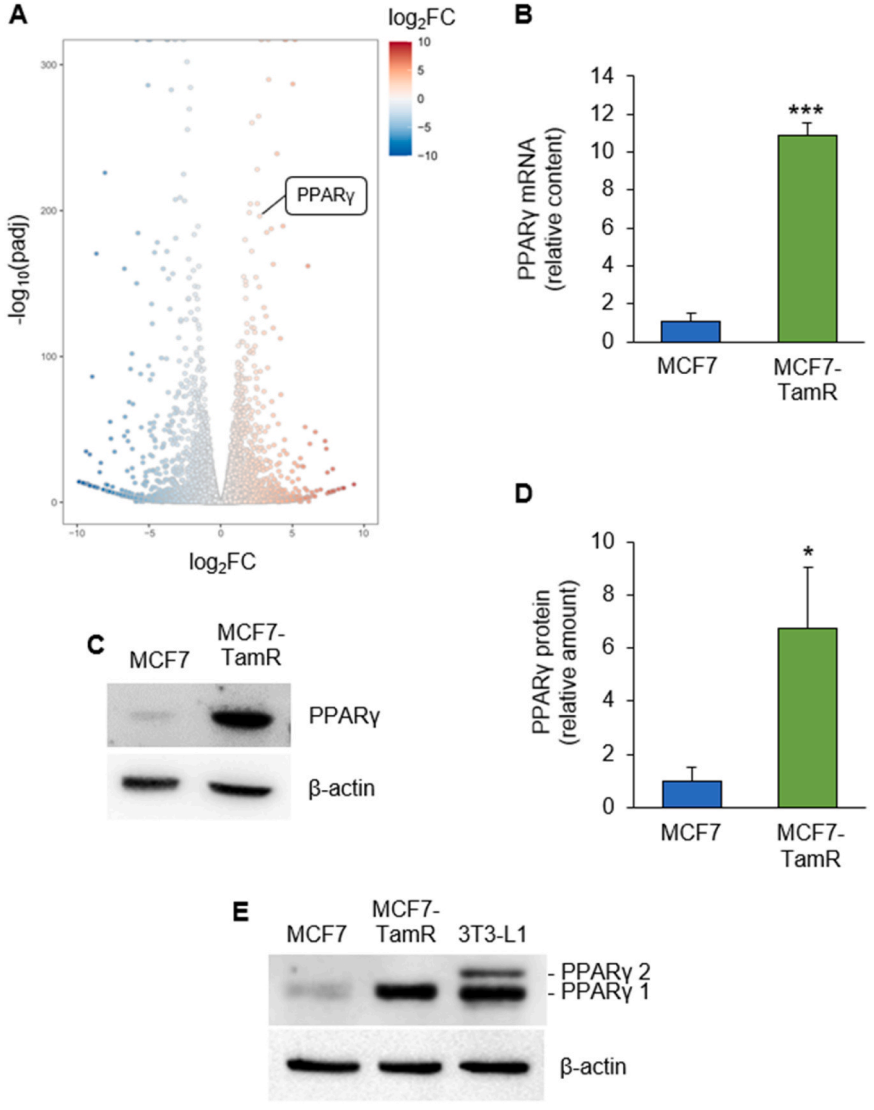


**Fig. 1.** PPARγ expression in MCF7 and MCF7-TamR cells. A. Volcano plot of the differentially expressed genes in MCF7-TamR *vs* MCF7 cell lines. The up- or downregulated genes are shown in red or blue, respectively; color intensity is proportional to the fold change. B. RT-qPCR validation of the RNA-seq data for PPARγ. C. Representative blot and (D) quantification of relative content of PPARγ protein. E. PPARγ isoforms expressed in MCF and MCF7-TamR cells. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with the Student's *t*-test: *: $p < 0.05$; ***: $p < 0.001$.

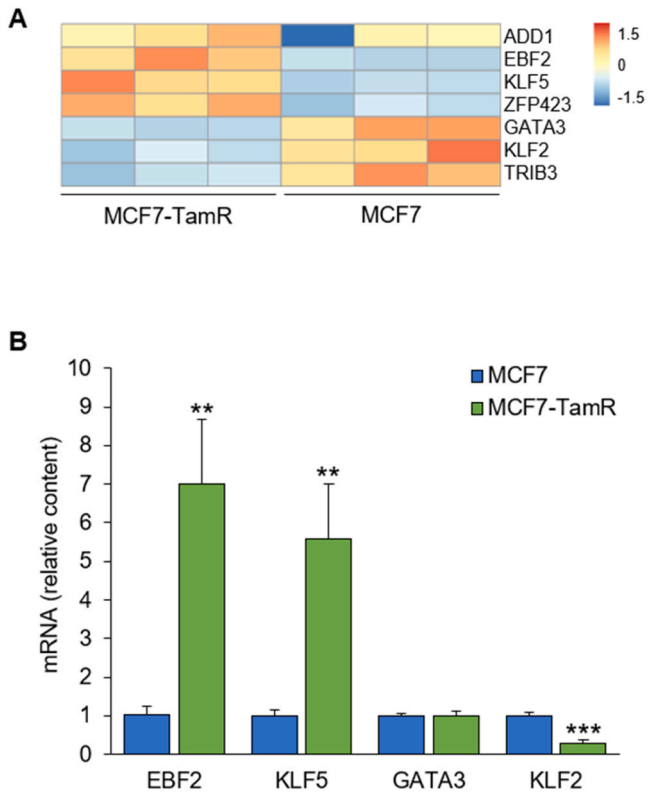


**Fig. 2.** Modulation of PPARγ transcriptional regulators in MCF7 and MCF7-TamR cells. A. Expression of selected transcription factors controlling PPARγ in MCF7 and MCF7-TamR cells based on RNA-seq data. Each column represents a different sample of the triplicate analyzed for the indicated cell line. B. RT-qPCR validation of RNA-seq data for the selected PPARγ regulators. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with the Student's *t*-test: **: $p < 0.01$; ***: $p < 0.001$.

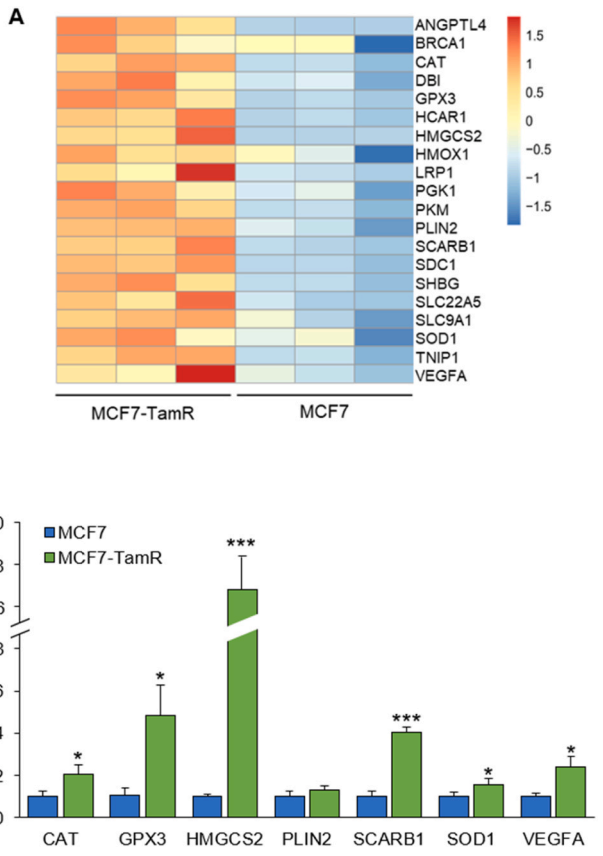


**Fig. 3.** Differential expression of representative PPARγ target genes in MCF7-TamR cells**.** A. Heat map of a selection of PPARγ target genes that are significantly upregulated in MCF7-TamR compared with MCF7 cells as from RNA-seq data. Each column represents a different sample of the triplicate analyzed for the indicated cell line. B. RT-qPCR validation of the RNA-seq data for some PPARγ target genes included in the heat map shown in A. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with the Student's *t*-test: *: $p < 0.05$; ***: $p < 0.001$.

included genes involved in important cellular processes, such as antioxidant defense (CAT, GPX3 and SOD), lipid synthesis and metabolism (DBI, HMGCS2, PLIN2 and SCARB1), angiogenesis and response to hypoxic conditions (VEGFA, ANGPTL4, HMOX1, PGK1, and PKM). For some of them, this information was further confirmed by RT-qPCR (Fig. 3B). These findings demonstrate that overexpression of PPARγ in MCF7-TamR cells increases the transcription of some of its target genes which control functions critical for cell survival.

### *3.4. Interfering with PPARγ expression or activity affects the growth and viability of MCF7 and MCF7-TamR cells*

The above results suggest that PPARγ activation and the ensuing upregulation of some of its target genes in MCF7-TamR cells could contribute to grant BC cells with a greater survival capability in hostile environmental conditions and in the presence of Tam. In keeping with this view, we have verified whether interfering with the expression or the activity of this transcription factor affects survival of parental and Tam-resistant MCF7 cells.

PPARγ was downregulated by exposing the cells to esiRNAs, which significantly reduced the intracellular amount of its mRNA and protein in both cell lines already after 36 h of treatment (Fig. 4A-C). PPARγ silencing effectively reduced the viability of parental MCF7 cells and restored the susceptibility of the Tam-resistant subclone to the drug (Fig. 4D).

We subsequently verified whether and to what extent also the pharmacological inhibition of PPARγ with a validated antagonist affected the viability of parental and MCF7-TamR cells. For these experiments we selected GW9662, an irreversible PPARγ inhibitor [30]. As expected on the basis of the data gathered with the specific esiRNAs, the treatment with GW9662 for 96 h dose-dependently reduced the viability of both MCF7 and MCF7-TamR cells, adding further evidence that this transcription factor critically contributes to survival and Tam resistance of MCF7 and MCF7-TamR cells, respectively (Fig. 5A, B).

More recently Imatinib has gained attention for its capability to interfere with PPARγ activity [25,31]. Due to this property and to the fact that this drug is already approved for use in humans, we investigated the effect of Imatinib on survival and Tam resistance of MCF7-TamR cells. As expected based on the results gathered with GW9662, Imatinib brought about a dose-dependent reduction of the viability of both MCF7 and MCF7-TamR cells.

The above finding was further investigated by assessing the colony-forming capability of parental and Tam-resistant MCF7 cells following a single treatment with either GW9662 or Imatinib at the same concentrations used for the viability assays. After 14 days, GW9662 at both the concentrations used completely prevented the formation of colonies from both types of cells (Fig. 6A). Comparable results were obtained with Imatinib, where 20 and 40 μM of the drug totally prevented the development of colonies.

### *3.5. PPARγ antagonists GW9662 and Imatinib affect the viability of spheroids formed from both parental and Tam-resistant cells*

To further corroborate the above results, we used BC cell spheroids as a reliable 3D model of cancer cell growth *in vivo*. Remarkably, both

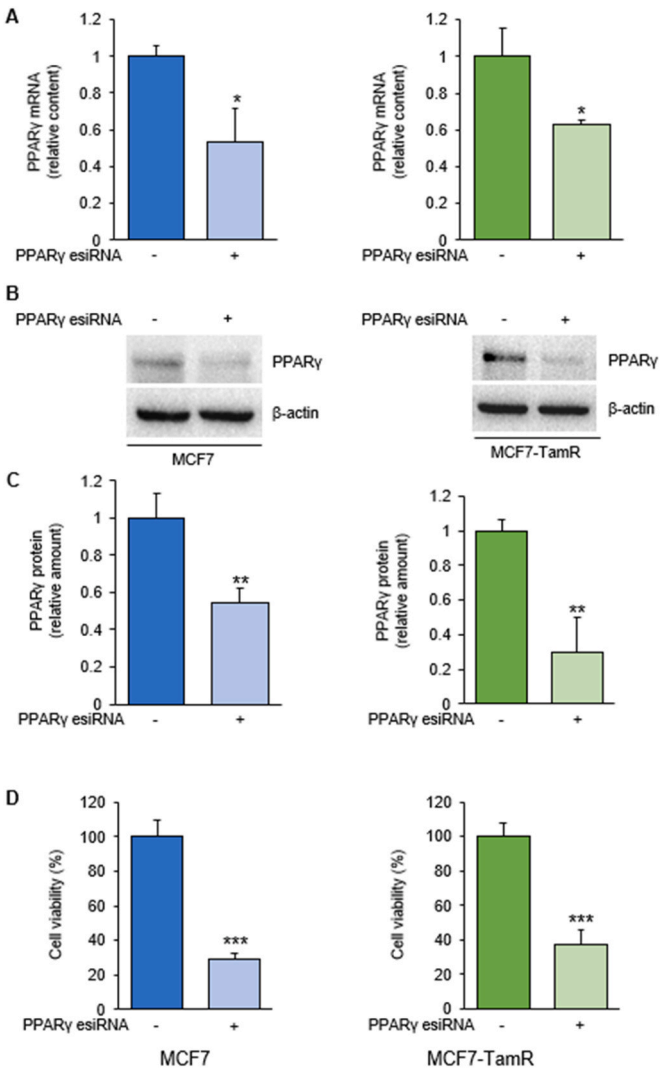


**Fig. 4.** Effect of PPARγ silencing on viability of MCF7 and MCF7-TamR cells. A. Relative amount of PPARγ mRNA after 36 hours of treatment with PPARγ esiRNA. B, C. Relative amount of PPARγ protein after 36 hours of treatment with PPARγ esiRNA. D. Effect of PPARγ silencing for 96 hours on the viability of MCF7 and MCF7-TamR cells. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with the Student's *t*-test: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

GW9662 and Imatinib induced a dose-dependent growth arrest and appearance of morphological alteration in the spheroids formed by both parental and Tam-resistant cells. The effect was evident already after 48 h of treatment with both drugs (Fig. 7A and B, respectively). After 96 h of incubation, the spheroid viability was assessed by PI staining. Whilst control or DMSO-treated spheroids presented only a few red-fluorescent cells, those treated with either GW9662 or Imatinib evidenced a dose-dependent increase of PI-positive cells, indicating the occurrence of extensive necrotic cell death.

### 3.6. *Overexpression of PPARγ is a frequent occurrence during the onset of Tam resistance*

The above results primed us to further investigate whether PPARγ upregulation represents a peculiarity for MCF7 cells or is a frequent event in the acquisition of Tam resistance of luminal A BC cells. To this aim, we generated additional Tam-resistant sublines starting from other commonly used luminal A BC cells, the MDA-MB-415, ZR-75-1 and T47D cells. The amount of PPARγ protein largely varied among the

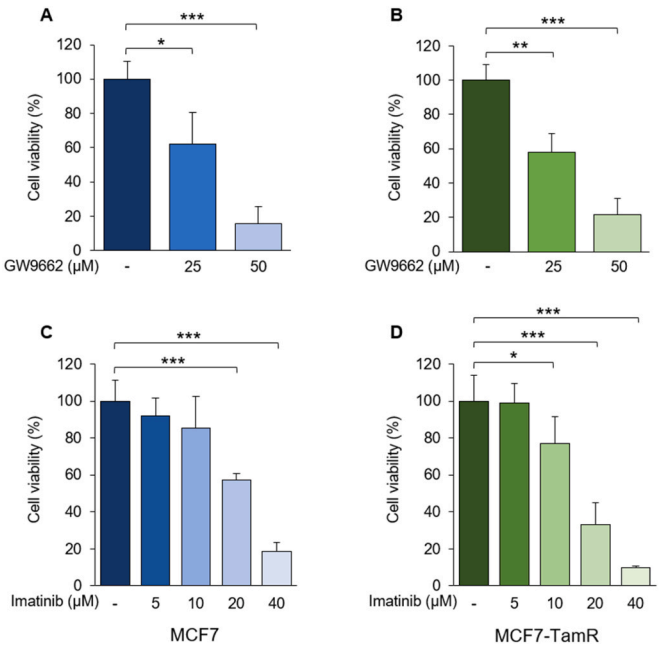


**Fig. 5.** Effect of GW9662 and Imatinib on cell viability. Viability of MCF7 and MCF7-TamR cells treated for 96 hours with the indicated concentrations of the PPARγ antagonist GW9662 (A, B) or Imatinib (C, D). Data represent the mean ± SD of three independent experiments. Imatinib: Imatinib mesylate. Statistical significance was assessed with one-way ANOVA followed by the *post-hoc* Dunnett's test: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

parental cell lines, and was the highest in the MDA-MB-415. Acquisition of resistance to 5 μM Tam did not modify the level of PPARγ protein of MDA-MB-415, but markedly increased that of ZR-75-1, whose intracellular content of PPARγ was about doubled. PPARγ protein was slightly increased also in T47D-TamR cells, without reaching the statistical significance (Fig. 8A, B).

The above cell lines were subsequently tested for their response to PPARγ inhibition by GW9662 or Imatinib. In agreement with the results gathered with MCF7 and MCF7-TamR cells, the viability of parental and Tam-resistant T47D and ZR-75-1 cells was markedly reduced by both GW9662 and Imatinib. By contrast, whilst the parental MDA-MB-415 cells showed a lower susceptibility to GW9662, the MDA-MB-415-TamR revealed to be totally irresponsive to the drug concentrations used in the present study (Fig. 8C). Imatinib markedly reduced the viability of all the tested BC cells, although both parental and Tam-resistant MDA-MB-415 cells proved to be markedly less susceptible than the other cell lines also to this drug (Fig. 8D).

### 3.7. *PPARγ expression in clinical samples of BCs*

Given that PPARγ was found to be frequently upregulated in most of the Tam-resistant subclones obtained from selection of luminal A BC cells, we have investigated how this gene is modulated in human BCs. This aim was attained by interrogating the publicly accessible TCGA and GEO BC datasets.

First, we analyzed PPARγ gene and its regulation using -omics data generated from BCs of the five molecular subtypes derived from a cohort of 1084 patients and made publicly available in the frame of the TCGA Pan Cancer Atlas project [32]. The PPARγ gene was altered in about 1.66 % of cases, of which 1.2 % and 0.46 % were amplifications and missense mutations, respectively (not shown). The amount of PPARγ mRNA in the above samples was substantially decreased in all BC subtypes compared with the normal adjacent breast tissue. Of interest, the luminal A was characterized by the greatest amount of PPARγ mRNA among all the subtypes considered (Fig. 9A).

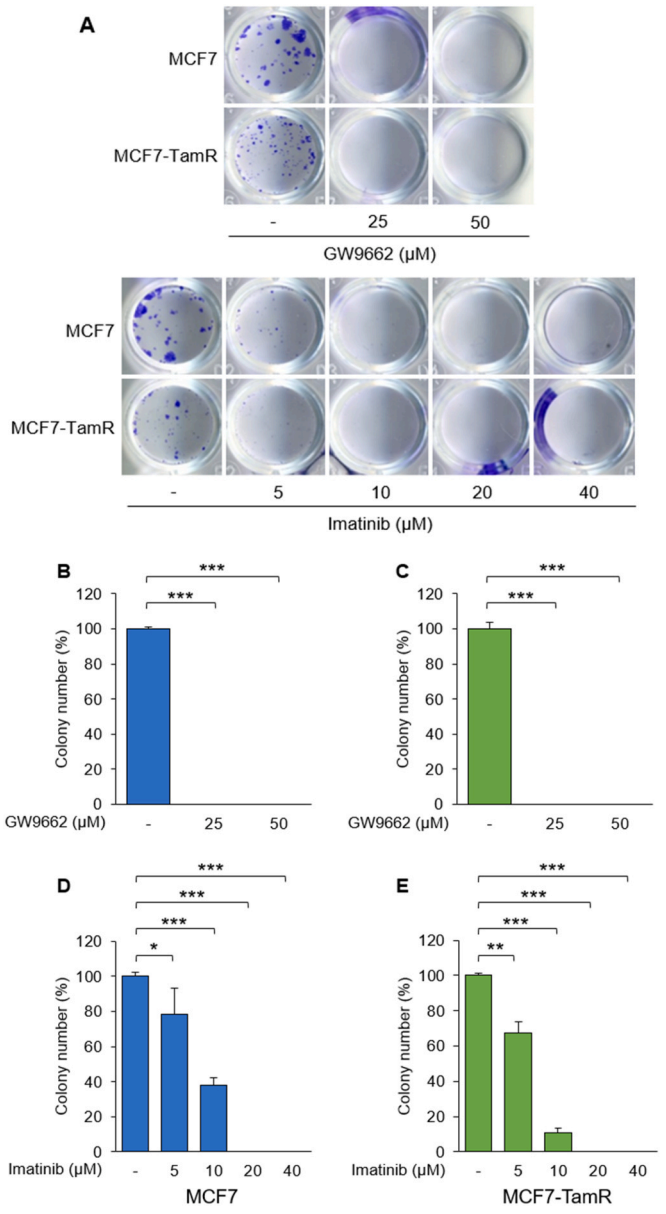


**Fig. 6.** Effect of GW9662 and Imatinib on the colony-forming capability of MCF7 and MCF7-TamR cells. A. Representative images of the colony formed by MCF7 or MCF7-TamR cells grown for 14 days after a single treatment with the indicated concentrations of GW9662 or Imatinib (upper or lower images, respectively). B-E. Number of colonies/well following treatment with GW9662 (B, C) or Imatinib (D, E) at the indicated concentrations. Imatinib: Imatinib mesylate. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with one-way ANOVA followed by the post-hoc Dunnett's test: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Furthermore, we also investigated PPARγ expression during BC progression *in vivo* by reanalyzing an RNA-seq dataset (GSE113890) generated from samples of normal peripheral blood (PB), circulating tumor cells (CTC) and metastasis (MT) collected from a small cohort of patients with advanced BC before starting a given therapeutic protocol or before therapy switch due to disease progression [27]. The relative number of PPARγ transcripts per million of reads was greater in CTC (baseline and follow-up) and metastasis than in PB samples collected before therapy application or at therapy switch (Fig. 9B), suggesting that dysregulation of PPARγ might play an important role in progression of luminal A BCs.

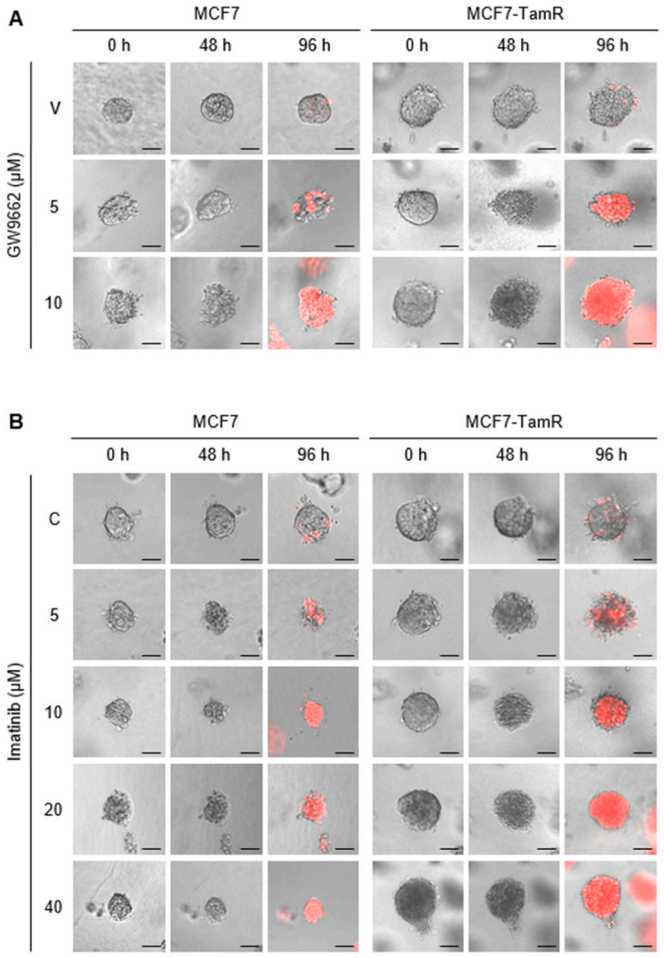


**Fig. 7.** Effect of GW9662 or Imatinib on the viability of spheroids generated by MCF7 and MCF7-TamR cells. Representative images of spheroids formed from MCF7 and MCF7-TamR exposed for up 96 h to the indicated concentrations of the PPARγ antagonist GW9662 (A) or Imatinib (B). The point '0 h' represents spheroids at the start of treatment with GW9662 or Imatinib. PI staining was performed only after 96 h of drug treatment. Imatinib: Imatinib mesylate. Scale bars = 50 μm.

## 4. Discussion

Acquisition of resistance to anticancer drugs is accounted for by deep alterations of gene regulation and metabolism of cancer cells [33–35]. The comparative transcriptomic analysis of a newly-generated subline of MCF7 cells resistant to 5 μM Tam and of its parental counterpart performed in this research has confirmed that the acquisition of Tam resistance is accompanied by a deep genetic reprogramming, manifesting with several differentially expressed genes (DEG) between the two cell lines. According to previous reports [36–38], several of the DEG identified in our study belong to pathways controlling cellular lipid metabolism, which is reported to be frequently dysregulated in carcinogenesis and likely represents one of the critical steps for the acquisition of endocrine resistance. In particular, our results have revealed that PPARγ, a transcription factor known to be the master regulator of lipid metabolism, is one of the most significantly upregulated genes in MCF7-TamR cells. The analysis of factors involved in the regulation of PPARγ evidenced that its overexpression stems from the convergent regulation of its positive and negative transcriptional modulators (significantly increased or decreased, respectively). The MRTFs have been recently reported to be part of a transcriptional feedback loop leading to PPARγ overexpression and increased synthesis of lipids, including fatty acids, phospholipids, and sphingolipids in the esophageal

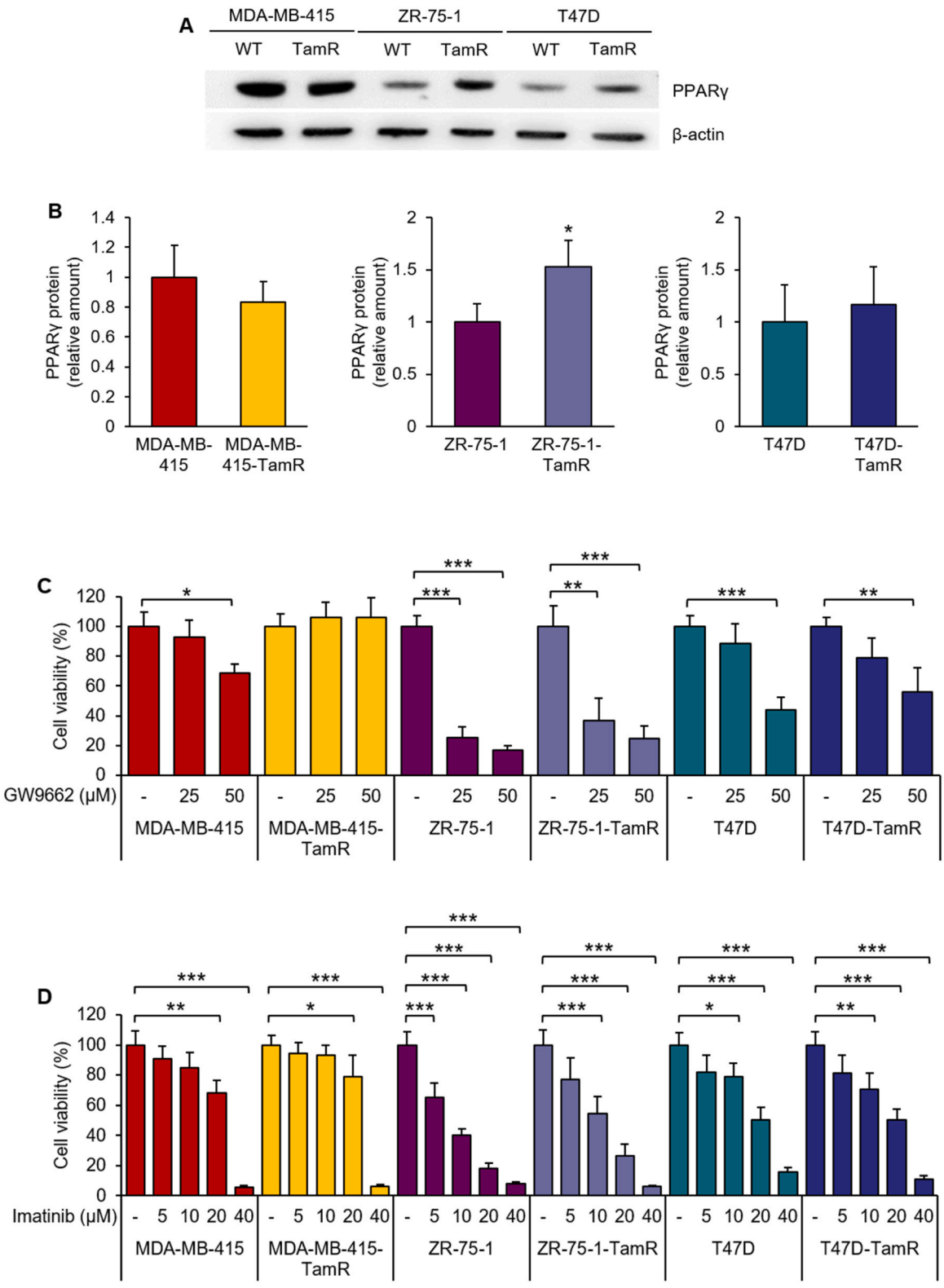


**Fig. 8.** Amount of PPARγ protein in luminal A breast cancer cell lines that have gained Tam resistance and effect of GW9662 and Imatinib on cell viability. Cellular content of PPARγ (A) and relative quantification of the protein (B) in the indicated parental or Tam-resistant BC cell lines. C. Effect of treatment for 96 h with different concentrations of GW9662 (C) or Imatinib (D) on the viability of BC cell lines of the luminal A subtype. Imatinib: Imatinib mesylate. Data represent the mean ± SD of three independent experiments. Statistical significance was assessed with one-way ANOVA followed by the post-hoc Dunnett's test: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

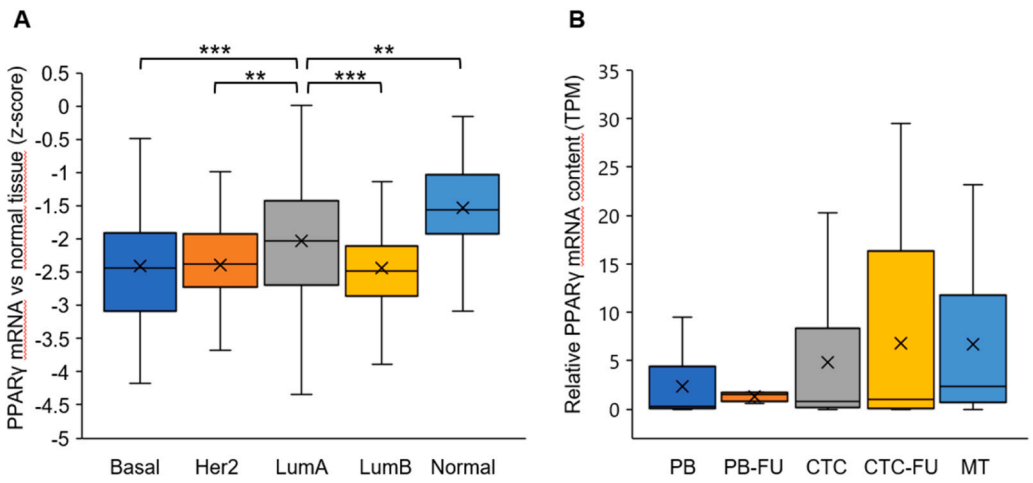


**Fig. 9.** Content of PPARγ mRNA in clinical samples of breast cancer. A. Amount of PPARγ mRNA in clinical samples of BCs belonging to the different molecular subtypes from a TCGA cohort of 1084 patients. B. Relative amount of PPARγ mRNA in peripheral blood cells, circulating tumor cells and metastases. Basal: triple-negative BC; Her2: HER2-overexpressing: LumA and LumB: luminal A or B molecular subtypes; Normal: Normal-like BC; TPM: transcripts per million of reads; PB: peripheral blood; CTC: circulating tumor cells: MT: metastasis; the suffix -FU indicates the relevant sample taken during the patient follow up. Data are expressed as z-score compared to the mean mRNA content of normal adjacent mammary tissue (A) or as the relative amount of mRNA versus the mean mRNA content of normal peripheral blood cells (B). Statistical significance was assessed with one-way ANOVA followed by the Tukey post-hoc test: **: $p < 0.01$; ***: $p < 0.001$.

adenocarcinoma [39]. The present research for the first time suggests the possibility that the MRTF/PPARγ axis plays a pivotal role also in determining or supporting BC progression and acquisition of endocrine resistance. Among the other factors known to negatively regulate the expression of PPARγ, TRIB3 gene was significantly downregulated in MCF7-TamR cells, a fact that might further contribute to PPARγ overexpression [40].

Bioinformatics analysis confirmed that, in Tam-resistant cells, PPARγ overexpression upregulates some of its major transcriptional targets. Their increased expression likely contributes to the induction of crucial events known to lead to cancer progression, such as the acquisition of angiogenic phenotype, invasiveness and resistance to oxidative stress. In addition to the above reported role in lipid metabolism, PPARγ has revealed also to regulate other biological functions. In preadipocytes, for instance, PPARγ slows down cell proliferation favoring their terminal differentiation into mature adipocytes [41]. Since ligand-mediated PPARγ activation negatively controls the growth of normal cells, this transcription factor has been endowed with a putative tumor-suppressor function [42–44]. In partial agreement with such a possibility, high expression or the prevalent nuclear positivity for PPARγ is considered a positive prognostic factor for luminal BCs [45]. Both the reanalysis of transcriptomics data from TCGA samples described in the present research, as well as previous data from other research groups converge on the finding that its expression is reduced in clinical specimens of primary BCs. This observation has stimulated intense investigation and great expectancy on the possibility that some of the approved PPARγ agonists, mostly used as antidiabetic drugs in the treatment of type 2 diabetes mellitus, can be effective also as antitumor agents. However, the experimental investigations and the clinical trials to test this hypothesis have produced unclear or frustrating results [22,29]. Based on the above paradigm, loss of PPARγ expression or activity due to deletion, silencing or transcriptional inactivation should favor or increase the frequency of BC development. In spite of these premises, transgenic mice with inactivated PPARγ did not show an increased tendency to develop spontaneous BCs [46]. However, upon carcinogenic treatment with DMBA, the transgenic PPARγ-silenced mice evidenced a greater incidence of faster-developing BCs than control mice. In particular, the tumors formed in the transgenic mice were mostly ER-positive ductal cancers, reminiscent of the luminal A molecular subtype in humans, which argues in favor of the existence of a peculiar link between PPARγ dysregulation and the BCs of the luminal subtype. This link seems to be confirmed by our results evidencing that increased expression of PPARγ is associated with the onset of Tam resistance also in most of the additional luminal A BC cells used, in addition to the formerly generated MCF7-TamR ones. The overexpression took place only in cell lines characterized by a low basal level of the transcription factor in the parental counterpart. No increase was observed when the parental cells already had high PPARγ content, suggesting that in these cells the expression of the transcription factor is not decreased during early phases of cancer development or that the development of endocrine resistance does not require a further upregulation of PPARγ.

The results of the present investigation strongly support the perspective that dysregulated PPARγ, other than promoting cancer progression in general, plays an important role in the acquisition of endocrine resistance of luminal A BC cells. Our observations agree with the results gathered from both clinical and experimental studies. A comparative transcriptomics analysis of drug-susceptible and resistant human BCs of the luminal A, B or basal subtypes evidenced that a set of 9 genes, which included PPARγ, could be considered reliable biomarkers of the acquisition of drug resistance [47]. Furthermore, our reanalysis of RNA-seq data from samples of BC patients in different clinical stages [27] has revealed that the amount of PPARγ mRNA was elevated in circulating tumor cells, and that its amount was comparable to that of metastases. PPARγ was also previously found to be upregulated in a subline of MCF7 cells resistant to 3 μM Tam. In these cells, its overexpression triggered the increase of cytokeratin-20 which, in turn, accounted for the greater invasiveness of Tam-resistant compared to parental MCF7 cells [48]. Eventually, the PPARγ ligands docosahexaenoyl ethanolamine and eicosapentaenoyl ethanolamine stimulated autophagy in MCF7 cells, providing them with a greater resistance to stresses, including those generated by treatment with anticancer drugs [49].

In the present research, the involvement of PPARγ in contributing to the onset of resistance to Tam in luminal A BC cell lines was confirmed by interfering with its expression or activity. The antagonist GW9662 was able to restore Tam susceptibility in cells in which the acquisition of Tam resistance is accompanied by an increased expression of PPARγ. Cytotoxicity of GW9662 exerted also on parental cells demonstrates that the inhibition of PPARγ, even if expressed at low level, can decrease cell viability also in the absence of Tam, further revealing the critical need of a functional PPARγ for survival of luminal A BC cells.

Worthy of interest, our research shows for the first time that also

Imatinib, the well-known receptor tyrosine kinase inhibitor currently used to treat hematological malignancies and recently revealed to be also a PPARγ antagonist [25], is effective also as single agent in killing both parental and Tam-resistant BC cells used in the study. In particular, our results have evidenced that Imatinib was capable of reducing the viability not only of conventional 2D cell cultures, but also of spheroids formed from parental or Tam-resistant MCF7 cells, a more reliable in vitro model for predicting the *in vivo* efficacy of the anticancer drugs [50]. Based on its demonstrated effectiveness in inhibiting PDGF receptor signaling and cell growth also of BC cells, Imatinib has already been previously tested in BC cell lines [51,52] or used in clinical trials to treat advanced or metastatic BCs either as monotherapy [53] or in combination with other drugs [54–56]. However, in spite of its high effectiveness shown *in vitro* [57,58], Imatinib-based clinical trials did not produce significant improvement of the clinical parameters of patients as to recommend its introduction as a standard treatment. A factor that could at least in part explain the poor impact of Imatinib on the clinical outcomes of BC patients could be found in the fact that the majority of patients enrolled in the different clinical trials was quite heterogeneous, bearing advanced or metastatic BC of different molecular subtypes, irrespectively of their hormone receptor status. In fact, due to the established activity of Imatinib on the receptor tyrosine kinases, the most stringent inclusion criterion adopted was the positivity for c-kit or PDGF receptors, the molecular targets against which this drug was originally developed and purposed [59]. However, our results demonstrate that Imatinib is very effective in reducing the viability of all the parental and Tam-resistant luminal A BC cells used in this research, and could therefore find a preferential area of application in therapy of this specific molecular subtype.

## 5. Conclusions

The results achieved in this research demonstrate that dysregulation of PPARγ and of some of its physiological target genes is a frequent biological occurrence and a founder event in the processes that drive the onset of Tam resistance in BC cells of the luminal A subtype. Consequently, the demonstration of the effectiveness of interfering with PPARγ expression or transcriptional activity gathered from all the experimental models used in the present investigation raises the possibility that PPARγ antagonism may be regarded as a novel and valuable strategy to prevent or overcome endocrine resistance. Along this line of evidence, our findings therefore pave the way to further explore the possibility of repurposing Imatinib, an FDA-approved drug for other neoplastic diseases, for the personalized treatment of luminal A BC subtypes (and prospectively, also of others) characterized by a constitutively elevated PPARγ expression, or by its upregulation consequent to acquisition of endocrine resistance. Such an approach would thus further expand the therapeutic options for selected cohorts of BC patients.

## Research data

The RNA-seq data used for the present research have not been deposited as yet in any public repository.

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## CRediT authorship contribution statement

**Giuliana Muzio:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Cecilia Boretto:** Methodology, Investigation, Data curation. **Riccardo Autelli:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117461.

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